# Plantas Geneticamente Modificadas (PGMs): obtenção e biosseguranca

“Agricultura Transgênica

- Tópicos de Biotecnologia em Plantas 2020
- Professora Maria Lucia Carneiro Vieira
  - Material didático Departamento de Genética, ESALQ/USP

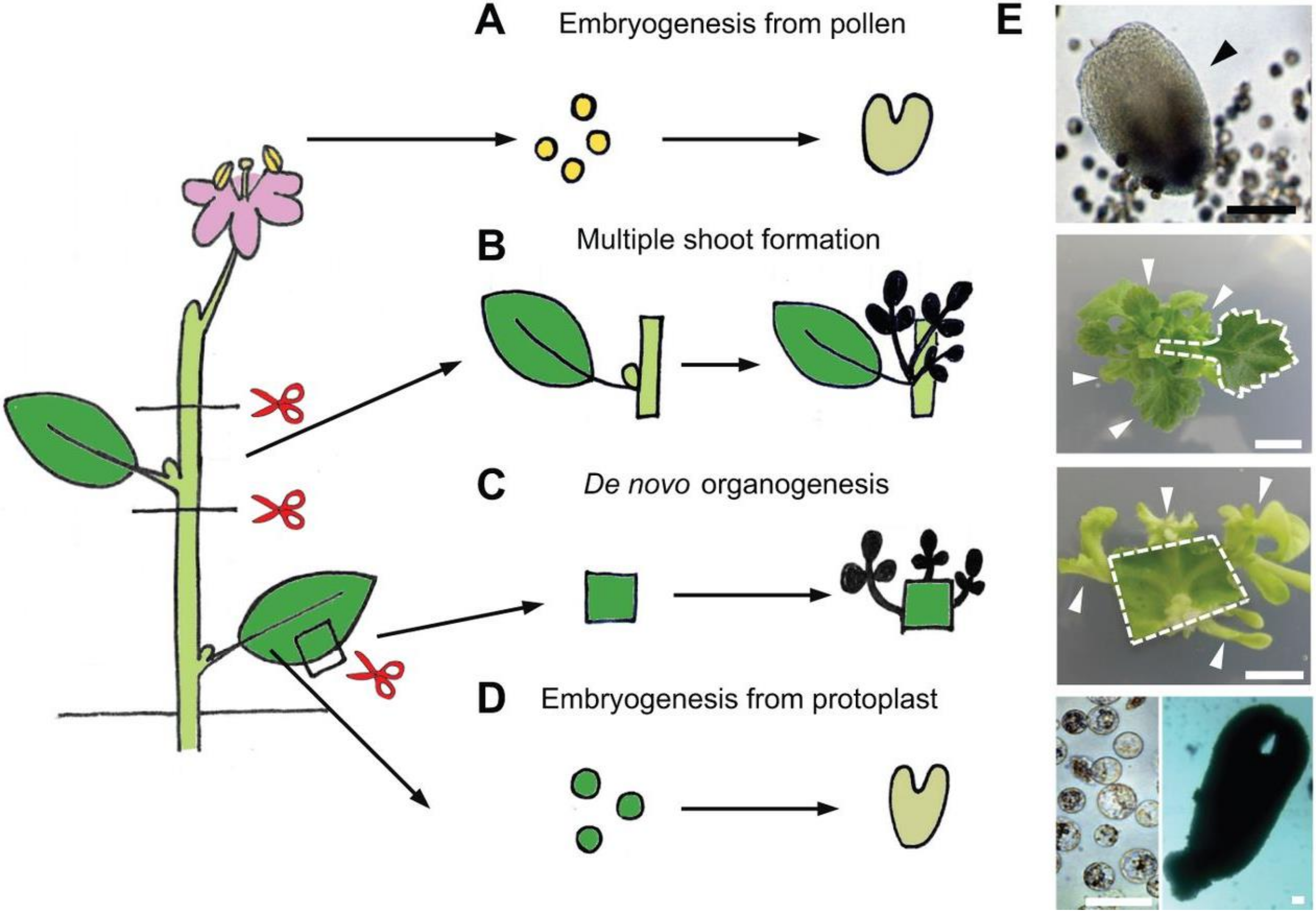
# Conhecimentos que propiciaram a adoção da transgenia

- O Melhoramento clássico levou à geração de variedades e híbridos elites
- Técnicas de cultivo *in vitro* (desde a década de 30): regeneração de brotos a partir de explantes
- A tecnologia do DNA recombinante propiciou a manipulação do DNA: clivagem (enzimas de restrição), ligação de fragmentos (DNA ligase), clonagem em vetores e transformação bacteriana (*E. coli*)
- Polimerização *in vitro* (a partir de *primers* complementares às sequencias do vetor de clonagem) e sequenciamento por Sanger
- Alinhamento de sequencias e a disponibilidade dos bancos de dados de sequencias
- Descoberta da PCR

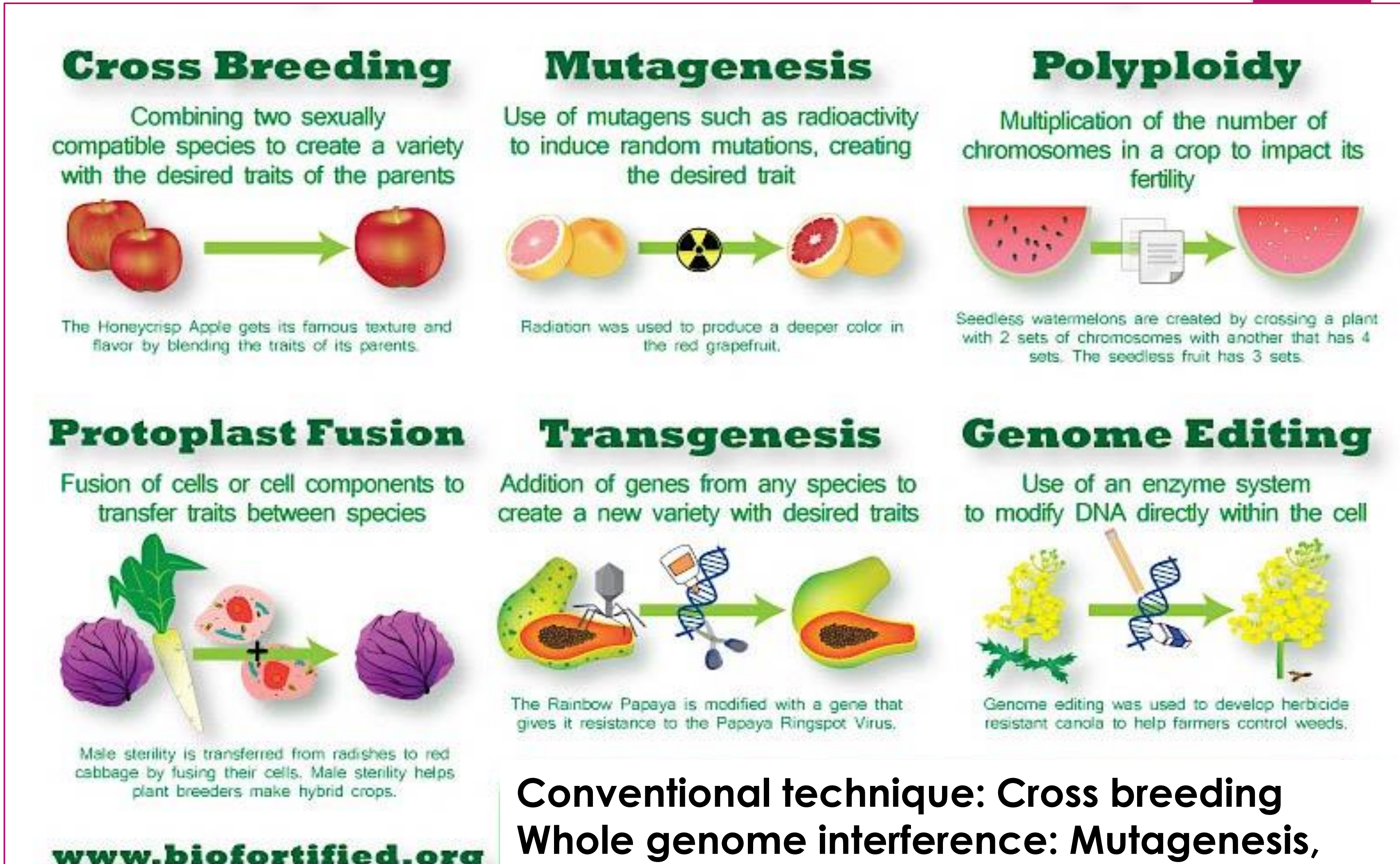
# *In vitro* Technologies

Compared with animals, plants generally possess a high degree of developmental plasticity and display various types of tissue or organ regeneration. This regenerative capacity can be enhanced by exogenously supplied plant hormones *in vitro*, wherein the balance between auxin and cytokinin determines the developmental fate of regenerating organs. Accumulating evidence suggests that some forms of plant regeneration involve reprogramming of differentiated somatic cells, whereas others are induced through the activation of relatively undifferentiated cells in somatic tissues.

- Plant regeneration: cellular origins and molecular mechanisms. Ikeuchi et al. ***Development*** 2016, 143: 1442-51; doi: 10.1242/dev.134668


A
Embryogenesis from pollen
B
Multiple shoot formation
C
De novo organogenesis
D
Embryogenesis from protoplast
E


García-Sogo B. et al. BMC Plant Biology (2012) (e-f). Production of somatic embryos in a callus of *Pelargonium peltatum* cultivated in selective **Morphogenesis Induction Medium (**MIM**)** (g). *P. peltatum* developing embryos in selective **Elongation Medium** (EM). (h). *P. peltatum* transgenic plantlets in **Rooting Medium** (RM). (i-j). Adventitious buds in a callus of *P. zonale* in selective MIM. (k). *P. zonale* shoot elongation in selective EM. (l). *P. zonale* rooted plantlet in RM. (m-n). Detection of transformation events in both *Pelargonium* spp.

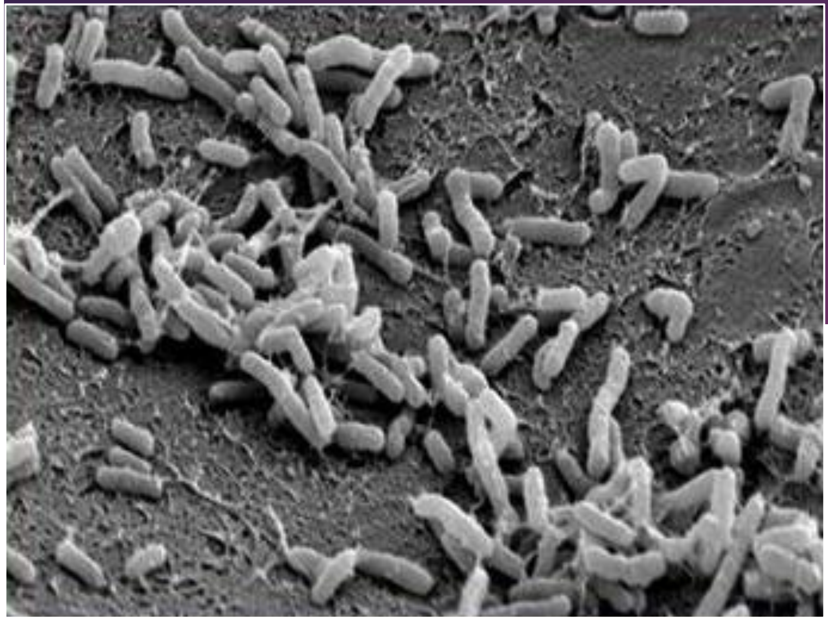



**Conventional technique: Cross breeding**
**Whole genome interference: Mutagenesis, Polyploidy induction and Protoplast fusion.**
**Molecular manipulation: Transgenesis, Genome editing**

# • Interação *Agrobacterium*-Planta
# • Introdução à Engenharia Genética de Plantas

Woody ornamental shrubs (rose), vines (grape), shade trees, fruit trees (cherry, berry, walnut), and herbaceous perennials

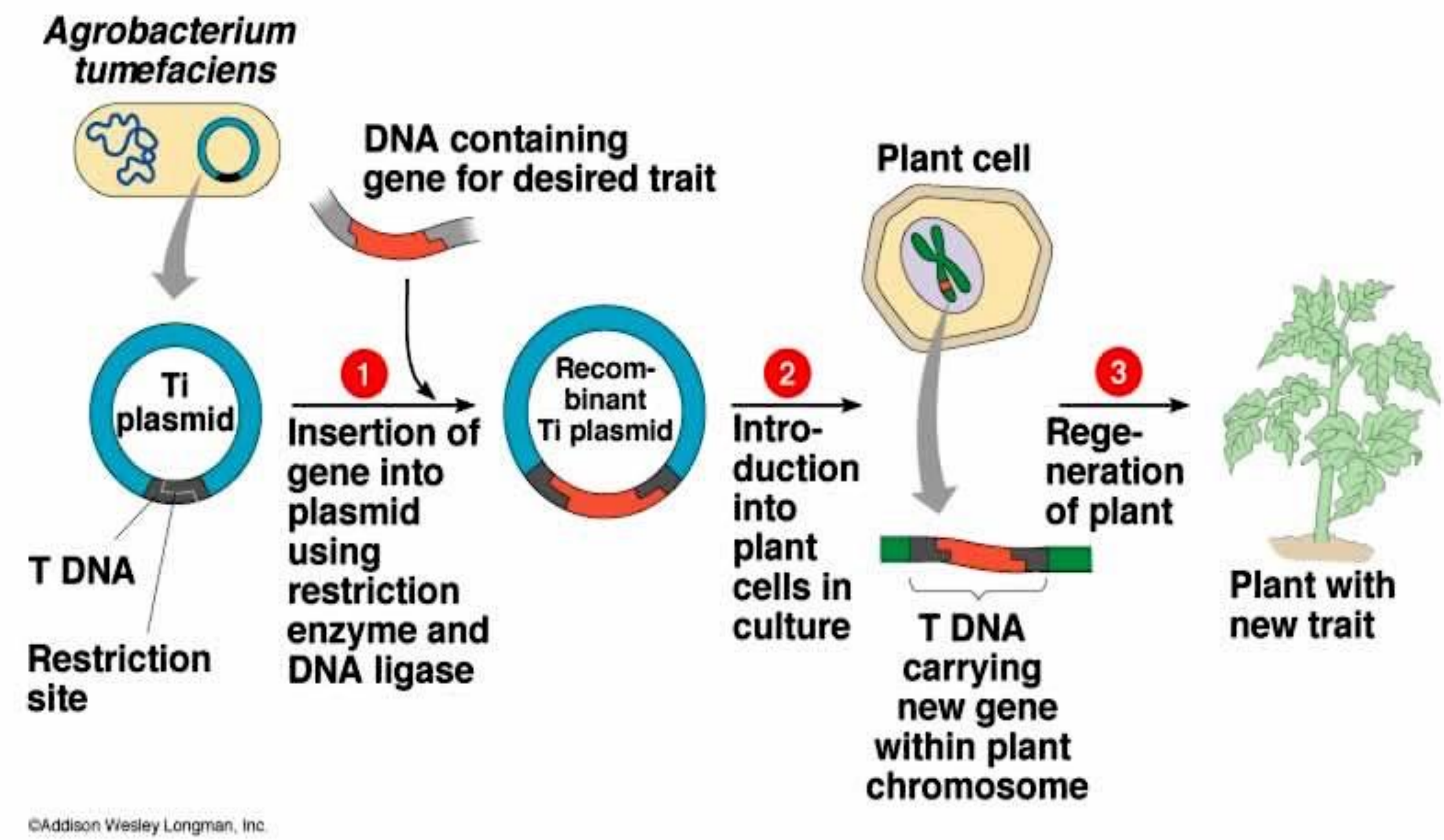

- *Agrobacterium tumefaciens* and related *Agrobacterium* species have been known as **plant pathogens** since the beginning of the XX$^{th}$ century. Since the first reports (1980s), scientists have attempted to improve this "natural genetic engineer" for biotechnology purposes.



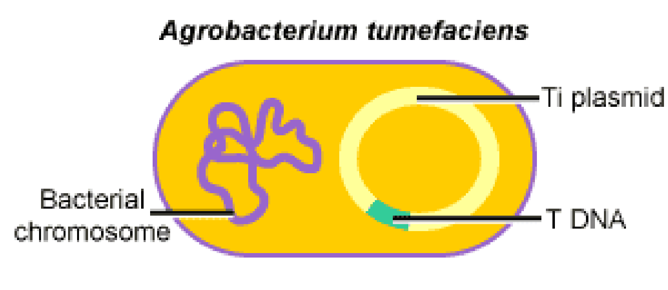
*Agrobacterium tumefaciens cells attached to a plant cell*

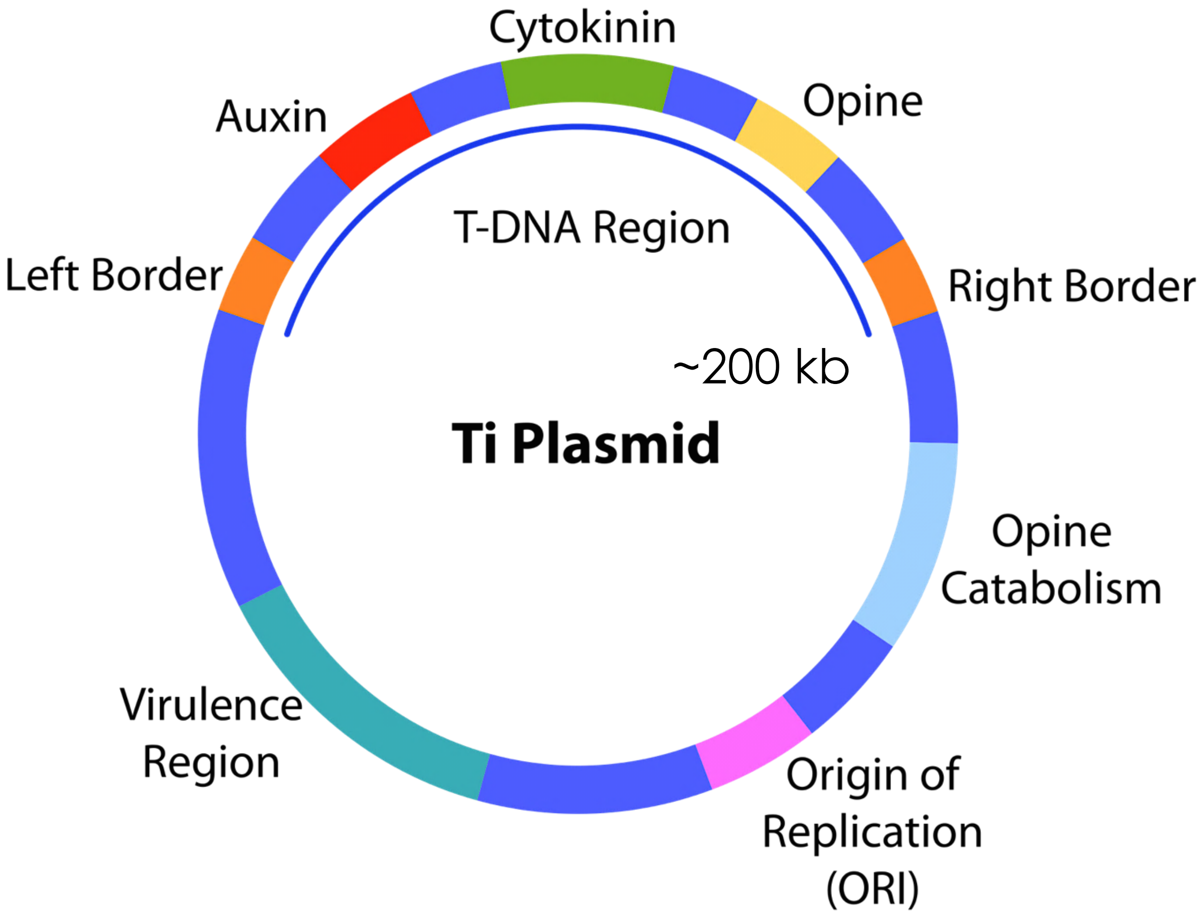

*Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species and gymnosperms.

# The unique ability of inter-kingdom DNA transfer: a genetic colonization



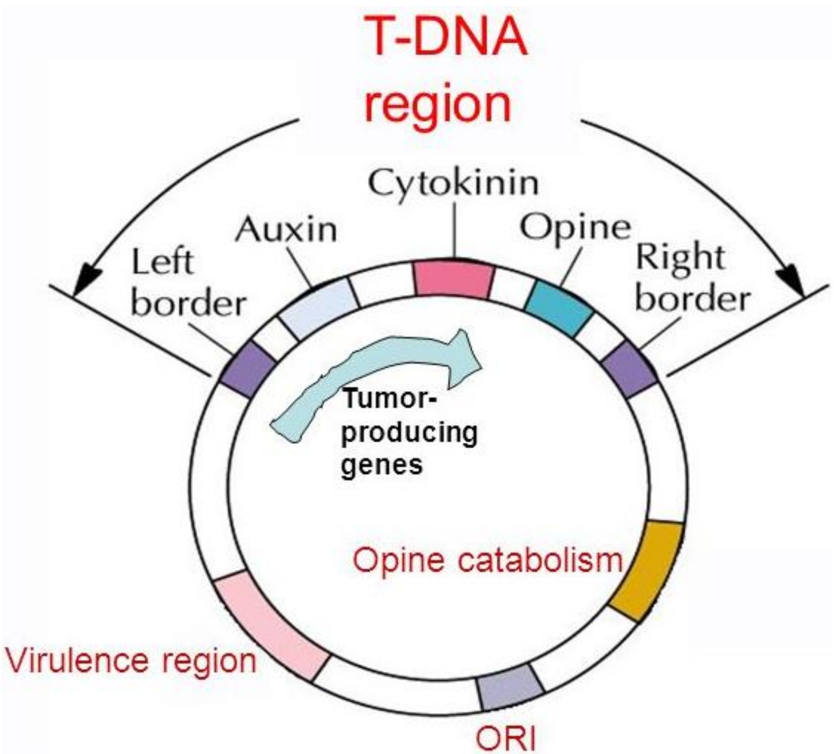
# Tumor-inducing (Ti) plasmid, which harbors a Transferred DNA (T-DNA)




Besides IAA and CK, infected plant cells produce over 20 kinds of opines, classified into 4 families: octopine, nopaline, mannopine, and agrocinopine families

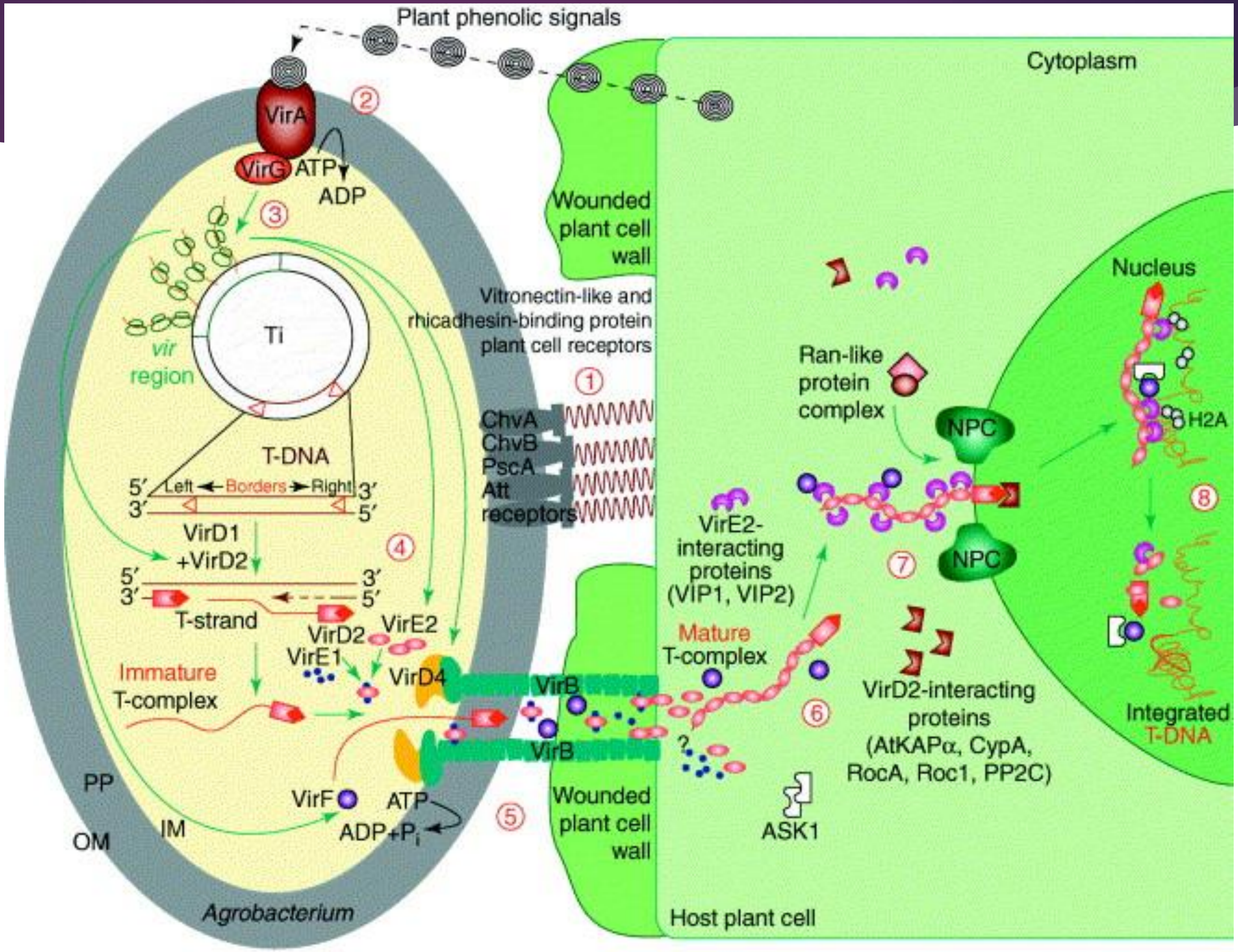
# • What is T-DNA?




Ti plasmids are on the order of 200 to 800 kp in size.

- T-regions on native Ti and Ri plasmids are 10 to 30 kp in size. Ti plasmids contain one T-region, whereas some contains multiple T-regions
- T-regions are defined by T-DNA border sequences (**25 bp in length and highly homologous in sequence**). They flank the T-region in a directly repeated orientation
- The border sequences not only serve as the target for the VirD1/VirD2 endonuclease but also serve as the covalent attachment site for VirD2 protein

# Molecular basis of the *Agrobacterium*-plant interaction

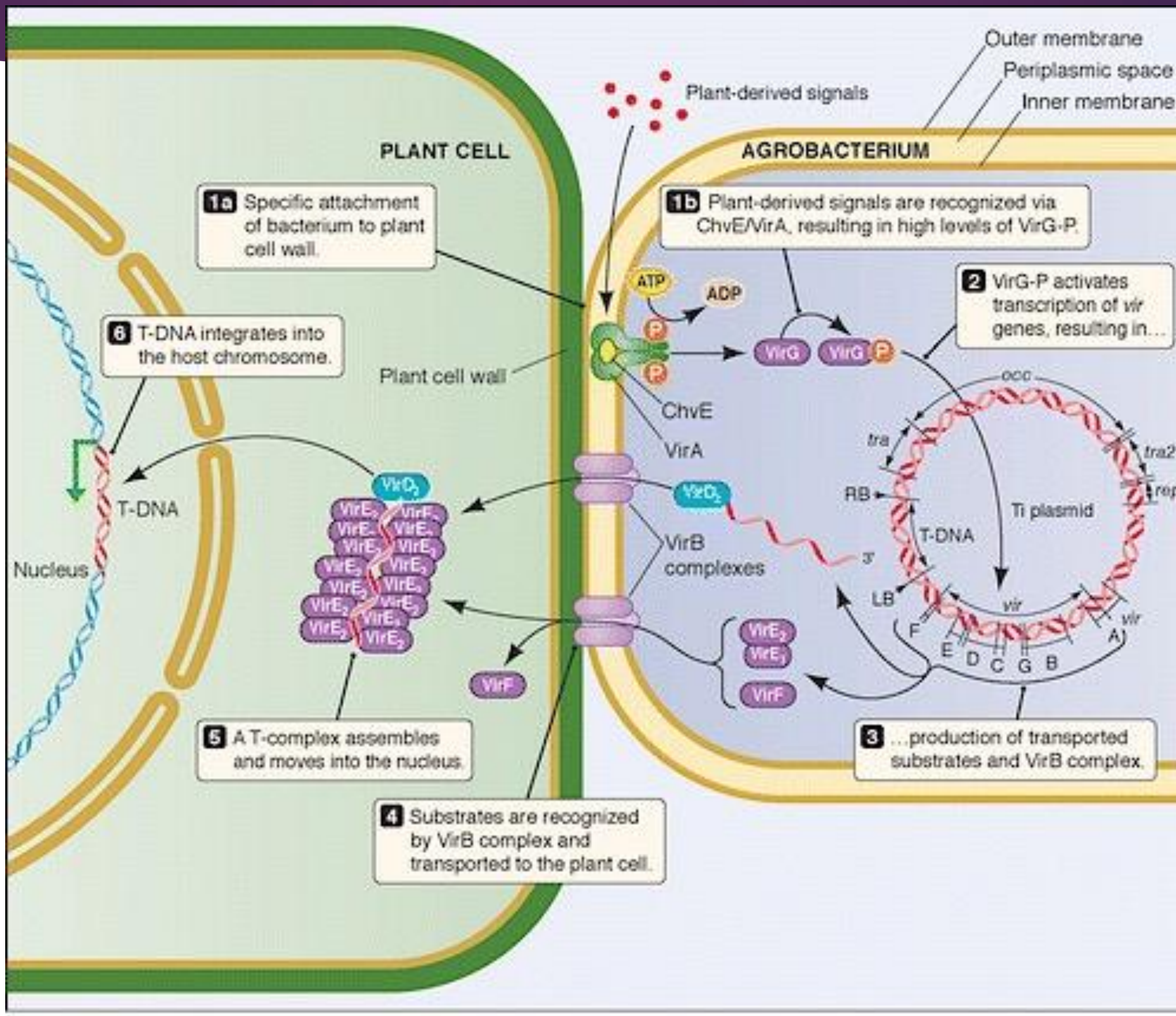



TRENDS in Cell Biology

- The Vir region on the Ti plasmid is a collection of genes whose collective function is to excise the T-DNA region of the plasmid and promote its transfer and integration into the plant genome

- The system is induced by signals produced by plants following wounding. Phenolic compounds such as acetosyringone, activate the Vir A gene, which is a constitutively expressed trans-membrane protein. The activated Vir A gene acts as a kinase, phosphorylating the Vir G gene. In its phosphorylated form, Vir G acts as an activator of transcription for the remaining Vir gene operons.
- Vir D1 + D2 have endonuclease activity, and make single-stranded cuts within the left and right borders. Vir E acts as a ssDNA binding protein, protecting the single strand T-DNA region during the transport phase of the process. Once in the plant cell, the complementary strand of the T-DNA is synthesized

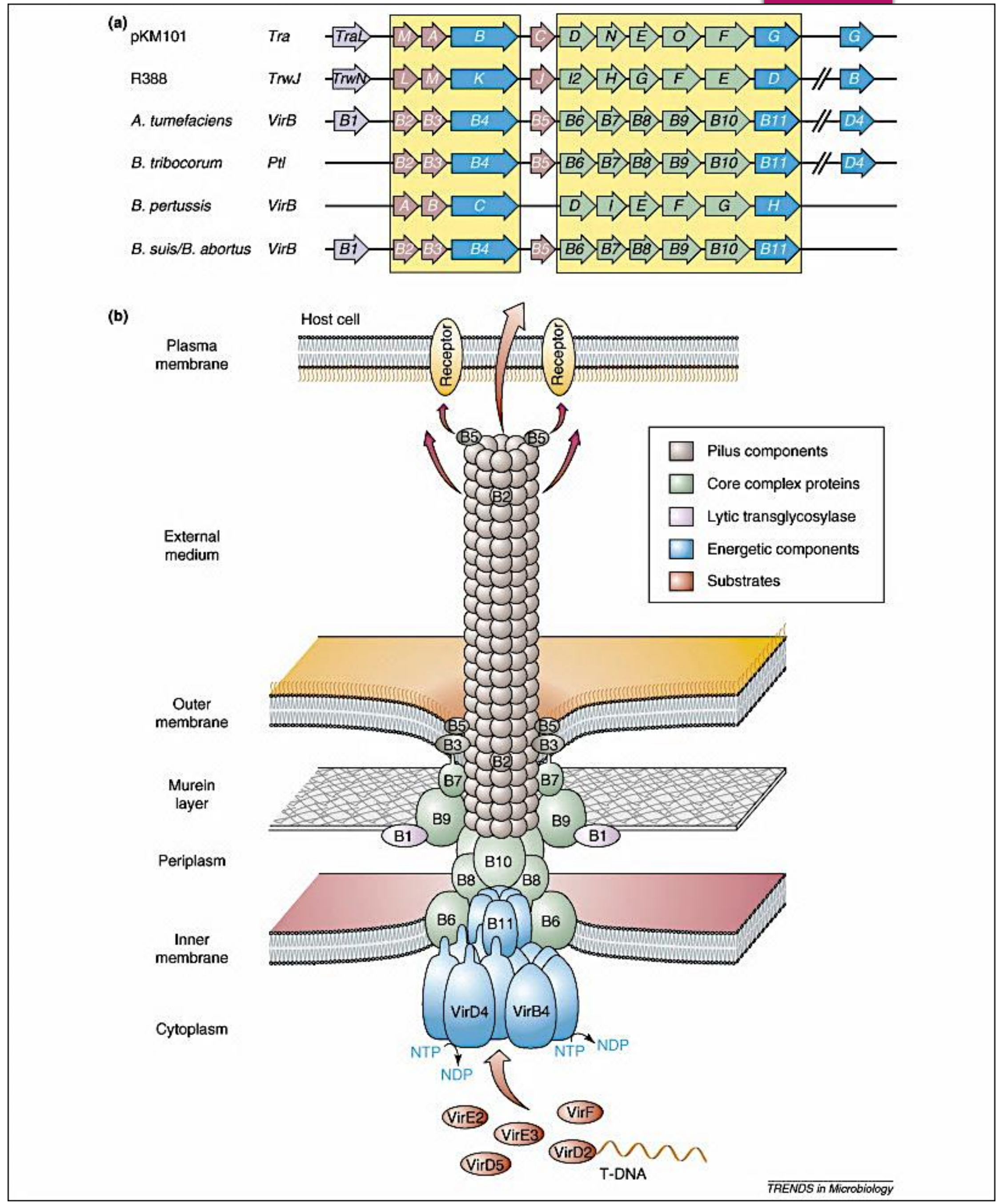
# *chv* genes play important roles in signal transduction necessary for *Agrobacterium* pathogenicity



- The 11 **Vir B** proteins make up <u>a type IV secretion system</u> to transfer the T-DNA and several other Vir proteins, including **Vir E2** and **Vir F.**

- Vir B proteins form the membrane channel or serve as ATPases to provide energy for channel assembly or export processes.

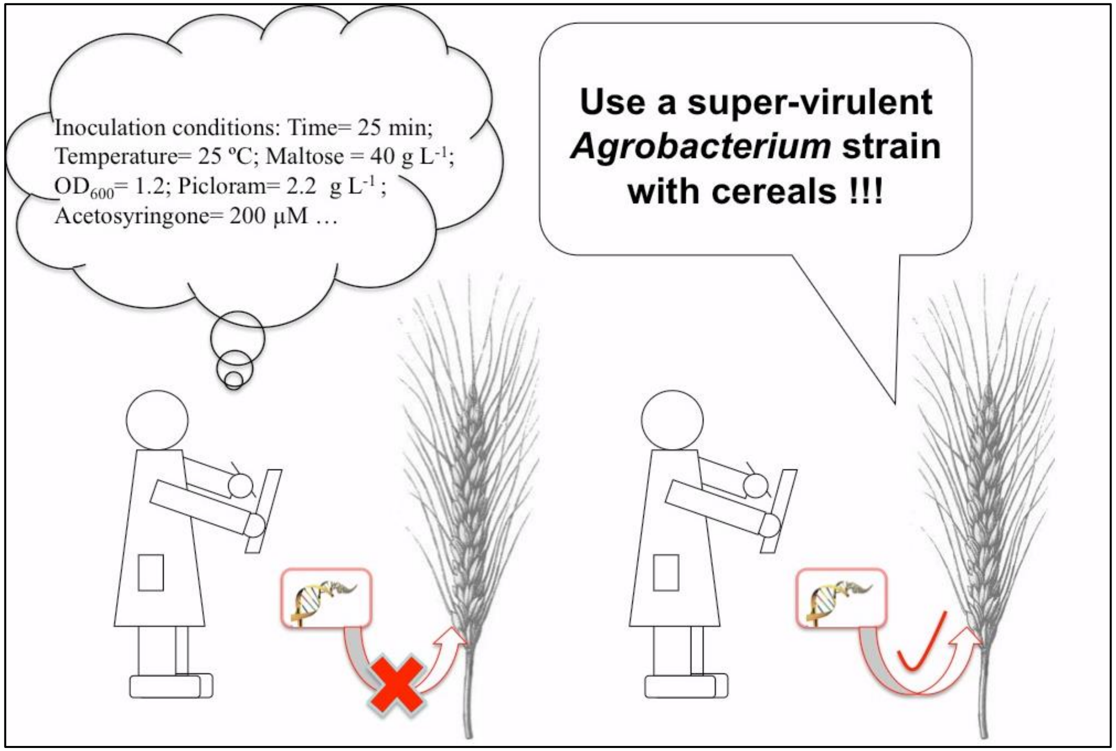
- Proteins, including Vir B2, B5, and B7, make up the T-pilus



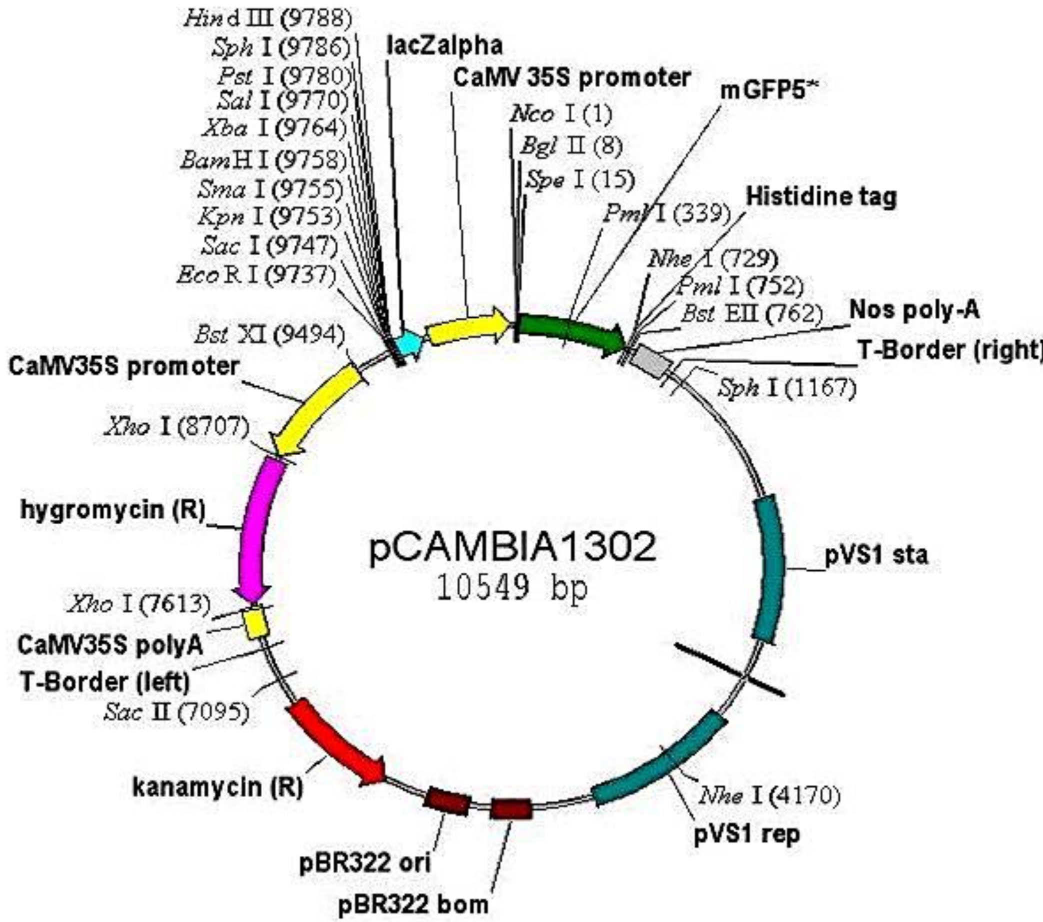
# Manipulation of *Agrobacterium* for Genetic Engineering Purposes

- Genetic experiments indicated that this particular class of plasmids, the Ti (and Ri) plasmids, were responsible for tumorigenesis and that the T-DNA is transferred to plant cells and incorporated into plant genome.

- It was obvious to propose that Ti plasmids should be used as a vector to introduce foreign genes into plant cells.

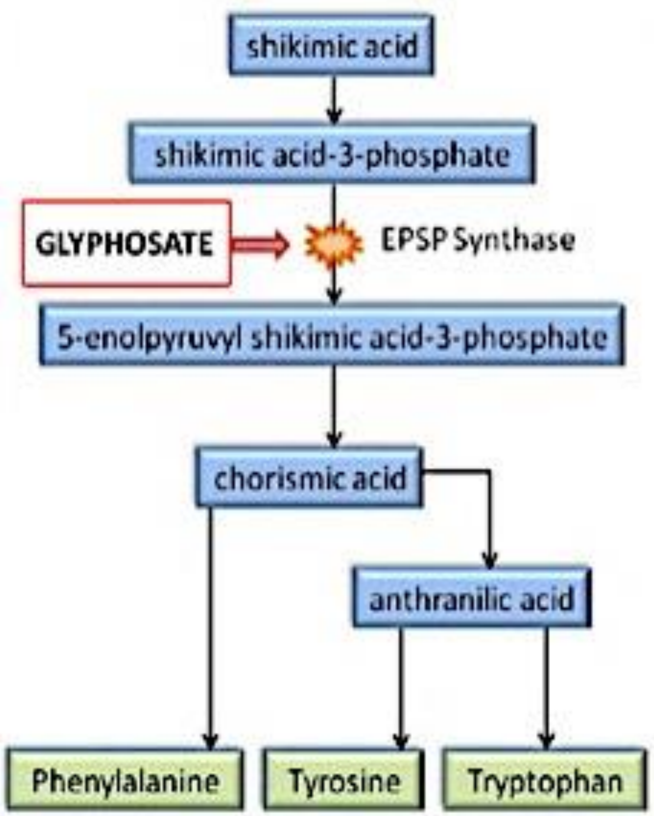


# • T-DNA based binary vectors




pVS1-REP and STA = regions for stability in *A. tumefaciens*

# ➢ Liberações comerciais de PGM no Brasil: Plantas tolerantes ao glifosato

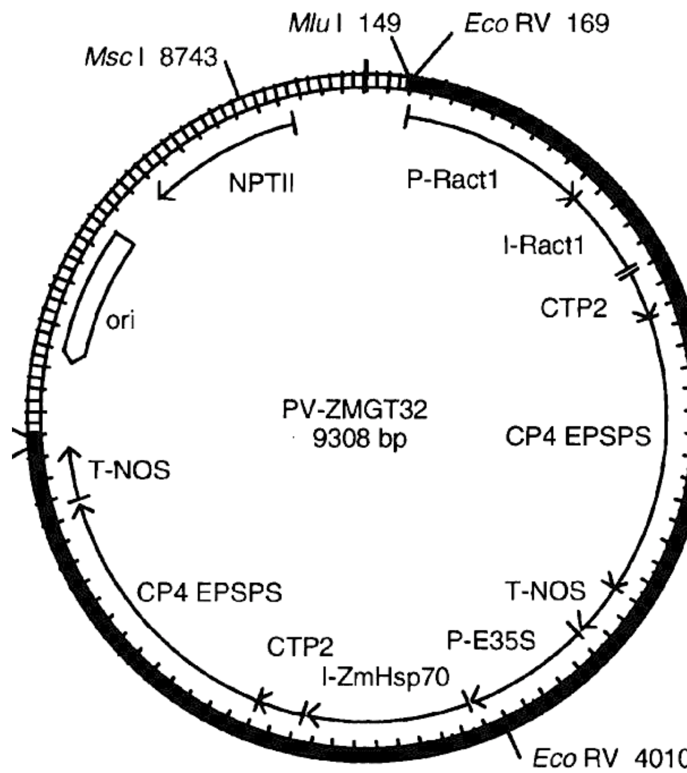



Gene *cp4epsps*, isolado da cepa CP4 de *Agrobacterium* sp., codifica a proteína CP4 EPSPS que confere a tolerância ao glifosato. A enzima EPSPS faz parte da via metabólica de biossíntese de aminoácidos aromáticos e compostos fenólicos. A ação do glifosato bloqueia essa enzima e desencadeia reações que levam a planta à morte. A **CP4 EPSPS** não é bloqueada pelo glifosato, permitindo o crescimento das plantas quando pulverizadas com o herbicida, já que a via de biossíntese é restaurada.

# Maize line NK603 is one of the events sold as "Roundup Ready". It contains two copies of the *cp4-epsps* gene transferring glyphosate tolerance

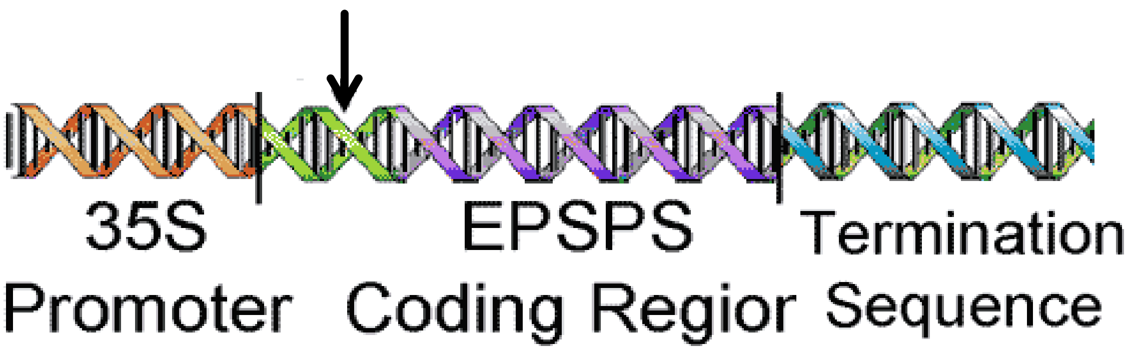
- The transgenes are controlled by **2 different promoters**, one of which the enhanced **CaMV 35S promotor**, the other a **rice actin promotor. Introns** associated with promoter sequences enhance expression in transformed cereals

- The event was developed by biolistic transformation using **one construct that contained both copies of the herbicide tolerance gene**.

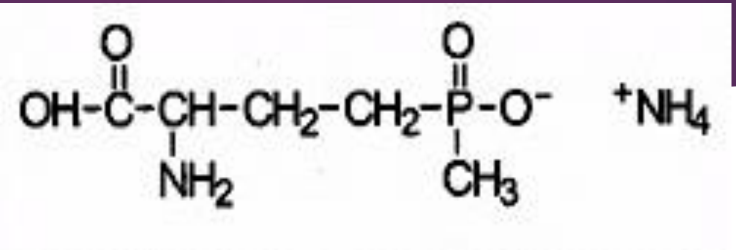


- Biolistic device used for direct plant transformation

- **Promoter**
- Chloroplast transit peptide (**CTP2**, isolated from *A. thaliana* EPSPS) for post-translational translocation of the CP4 EPSPS protein to the chloroplast
- *cp4 epsps* gene from *Agrobacterium* sp.
- 3' NOS transcriptional termination signal from *A. tumefaciens*
- EPSPS are proteins ubiquitous in nature



# ➢ Plantas tolerantes ao glufosinato de amônio




- Glufosinate-treated plants die as its active ingredient, **phosphinothricin**, **inhibits the activity of the glutamine synthetase enzyme by competitively binding in place of the normal substrate**, **glutamate (glutamic acid). This prevents the synthesis of *L*-glutamine**, which a chemical precursor for the synthesis of nucleic acids and proteins, and serves for ammonia incorporation for plants.

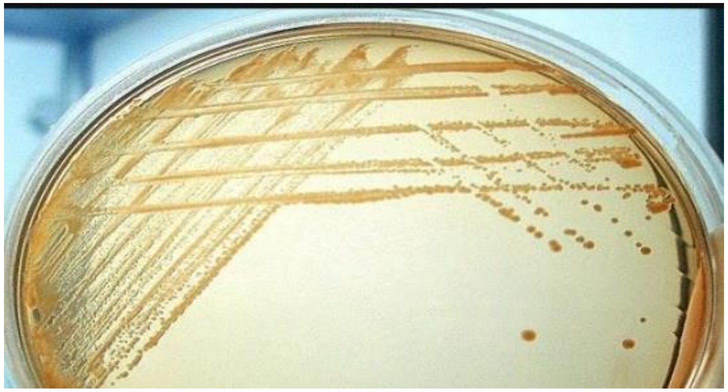
- The *bar* or *pat* gene, isolated from *Streptomyces* gives resistance as the PAT enzyme acetylates phosphinothricin at the N-terminus.

Plants transformed with the *bar* gene are resistant, and this is conferred through modification of the herbicide molecule.

The construct contains the **CaMV 35S promoter**, **t**he *pat* gene modified to increase its level of expression **and the CaMV 35S terminator.** The pUC sequences include an **ampicillin resistance gene** that has not functional regulatory signals in transgenic corn cells.

# ✓ Outras modificações visando a tolerância a herbicidas

- **Tolerância a 2,4-D: soja, milho (*aad-1*)**
- gene ***aad-12*** (de *Delftia acidovorans*): codifica a proteína ariloxialcanoato dioxigenase que catalisa a via de degradação do herbicida;
- **Tolerância a Dicamba: soja e algodão**
- gene **dmo** (de *Stenotrophomonas maltophilia*): enzima dicamba mono-oxigenase que usa o herbicida como substrato)



- **Tolerância aos herbicidas da classe das imidazolinonas e sulfoniluréias: soja**

  gene ***csr-1-2*** de *Arabidopisis thaliana*

- **Tolerância ao isoxaflutol: soja**

  gene ***hppd*** de *Pseudomonas fluorescens*

*Soja da Bayer Crop Science: "Balance GT 3": tolerante a glifosato, glufosinato de amônio e isoxaflutol*

# ➢ Plantas resistentes a insetos: genes *cry*

- (February 15, 2008) Brazil's National Biosafety Council gave the final clearance for 2 varieties of GM maize for commercial use. The varieties were Liberty Link, which is resistant to the herbicide glufosinate-ammonium and **insect-resistant MON 810**

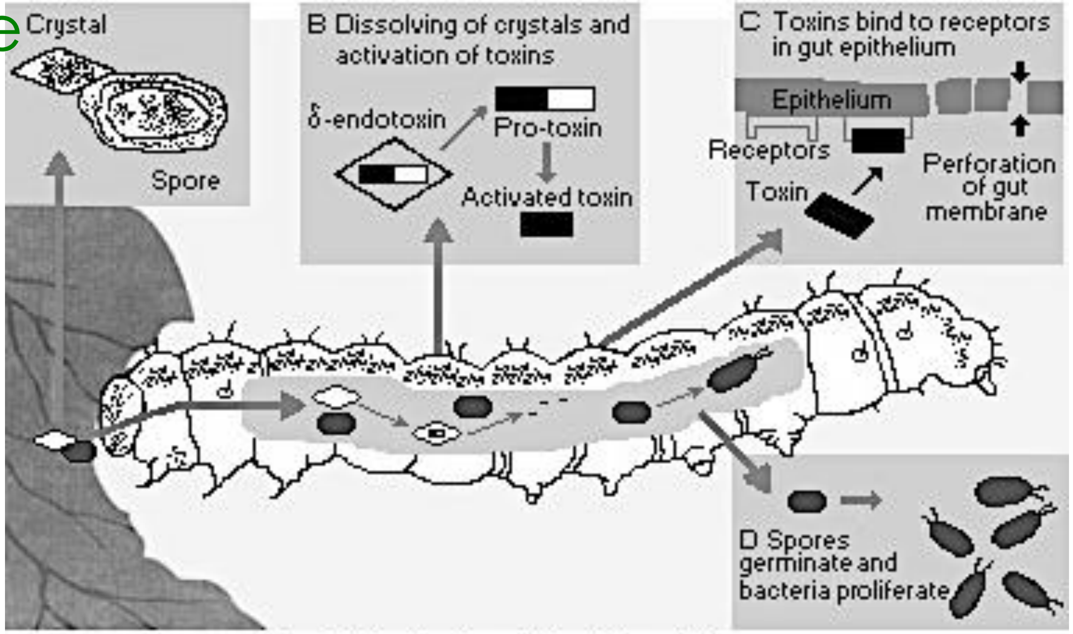
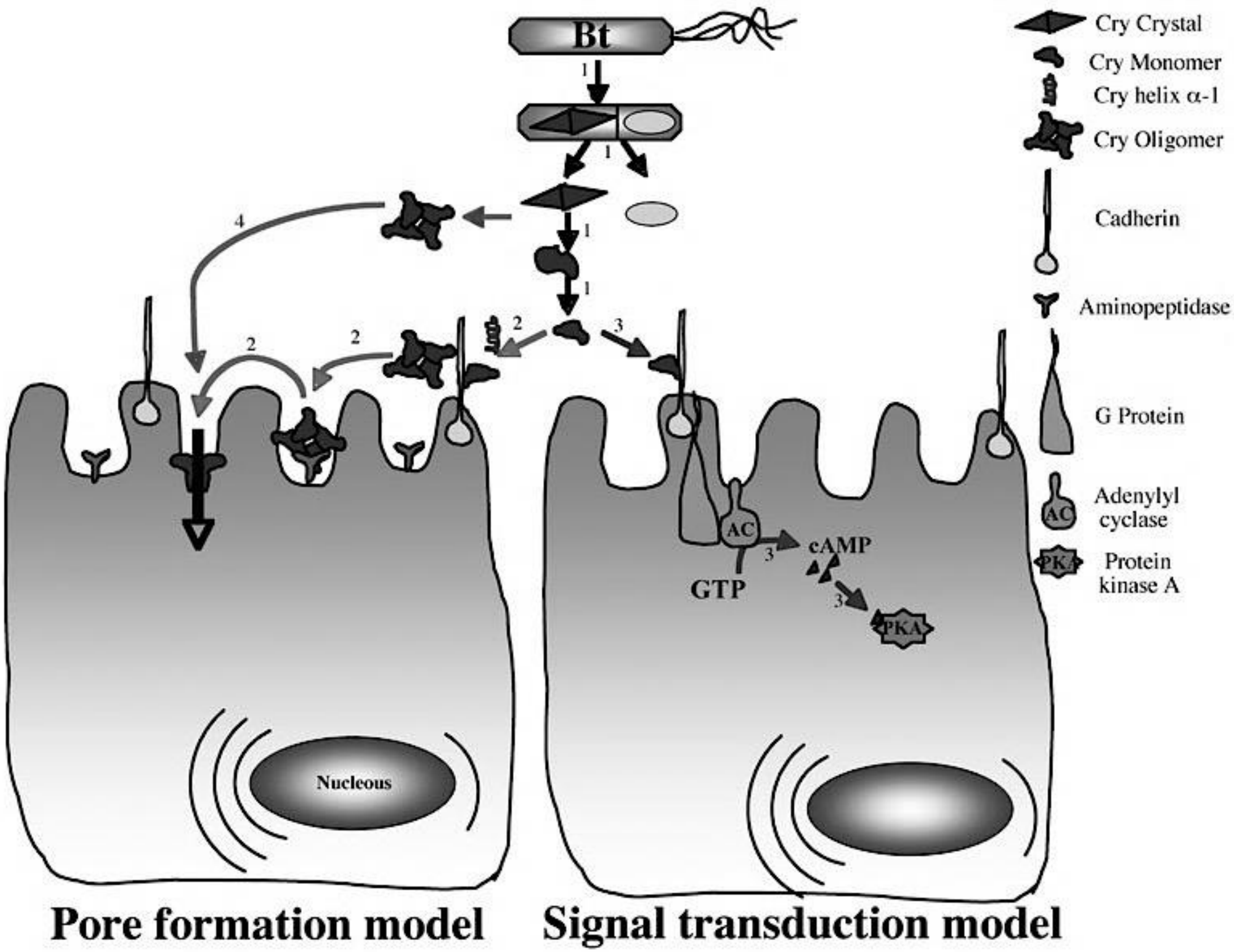



Fig. 1. Mechanism of toxicity of Bt

- Scientific literature support that a cadherin-like protein functions as a receptor for *B. thuringiensis* Cry1Aa and Cry1Ac toxins on mid-gut epithelial cells of insect larvae.



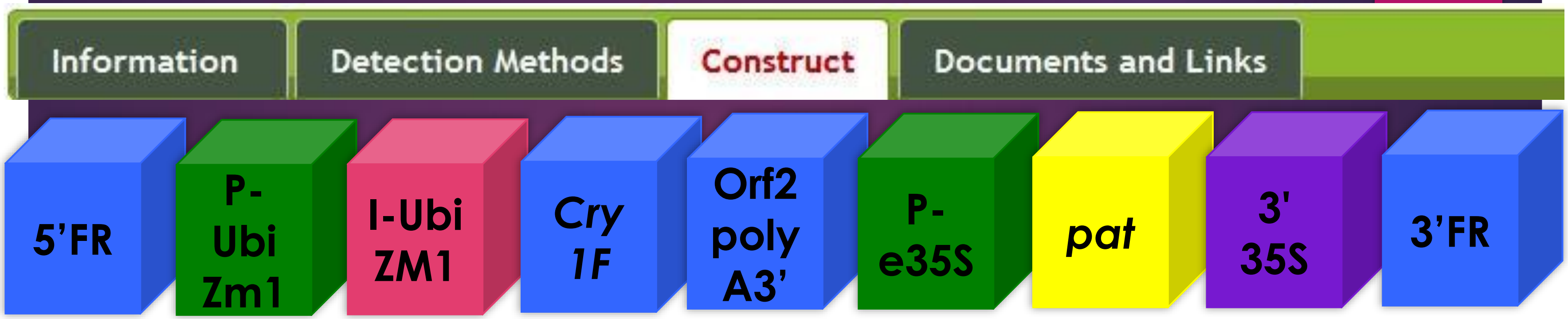
- MON810 contains a truncated portion of a synthetic form of the *cry1Ab* gene from *Bacillus thuringiensis*

- The technique used for the modification was **biolistic**. The construct contained the *cry1Ab* gene from *B. thuringiensis*, **which was modified to increase the levels of the Cry1Ab protein in plants**. An enhanced **CaMV 35S promoter** and the intron of the maize heat-shock protein were used to regulate the expression of the *cry1Ab* coding sequence. The **3' NOS transcriptional termination signal**, isolated from the Ti plasmid of *A. tumefaciens* was also part of the transformation cassette. **The plasmid also contained the *nptII* coding sequence used as selectable marker.**

# ➢ Combinando genes Cry

- **MON89034** foi transformado por *A. tumefaciens* e expressa os genes *cry1A.105* e *cry2Ab2*, que conferem resistência aos principais insetos que atacam as lavouras.  A proteína **Cry1A.105** resulta da expressão do gene *cry1A.105* que é uma construção quimérica dos genes *cry1Ab, cry1Ac* e *cry1F* e a **Cry2Ab** é uma variante da proteína selvagem, sendo ambas derivadas de *B. thuringiensis*. Estes genes foram inseridos em diversos eventos de **algodão** e **milho** gerados para plantio e consumo humano e animal.

http://en.biosafetyscanner.org/mostraevento.php?id=39




## COMPOSITION OF EVENT MAIZE TC1507

| Name | Description | Functions |
|---|---|---|
| 5'Frund | Undesired sequence at the 5' flanking region | -Not known |
| P-UbiZm1 | Constitutive promoter of ubiquitin 1 gene from Z. *mays* | -Promoter |
| I-UbiZM1 | Intron 1 of ubiquitin 1 gene from Z. mays | -Intron |
| Cry1F | Sequence encoding Bt endotoxin of *B. thuringiensis* var. azaiwai | -Insect resistance |
| Orf2 polyA3' | Bidirectional termination sequence from *A. tumefaciens* | -Polyadenylation site |
| P-e35S | Constitutive promoter of CaMV 35S gene | -Promoter |
| *Pat* | Sequence encoding PAT (phosphinothricin acetyl transferase) | -Glufosinate tolerance |
| 3' 35S | 3' termination sequence of the CaMV 35S gene | -Terminator |
| 3'FRund | Undesired sequence at the 3' flanking region | -Not known |

# ➢ Combinando fenótipos: algodoeiros GMs

| | |
|---|---|
| Widestrike/cotton | *Cry1F/Cry1Ac/pat* |
| BollGard/cotton | *Cry1Ac/Cry2Ab2* |
| MON531 x MON1445/cotton | *Cry1Ac/epsps* |
| BollGard/RR/Flex | *Vip3A, APH4, CryIAc, Cry2Ab2, CP4 EPSPS* |

- **RISK ANALYSIS OF STACKED EVENTS IS ALSO PERFORMED CASE BY CASE**
- **A DETAILED MOLECULAR CHARACTERIZATION OF THE STACKED GENES IS REQUIRED**
- **A FAST TRACK CAN BE FOLLOWED UNDER SPECIFIC SITUATIONS**

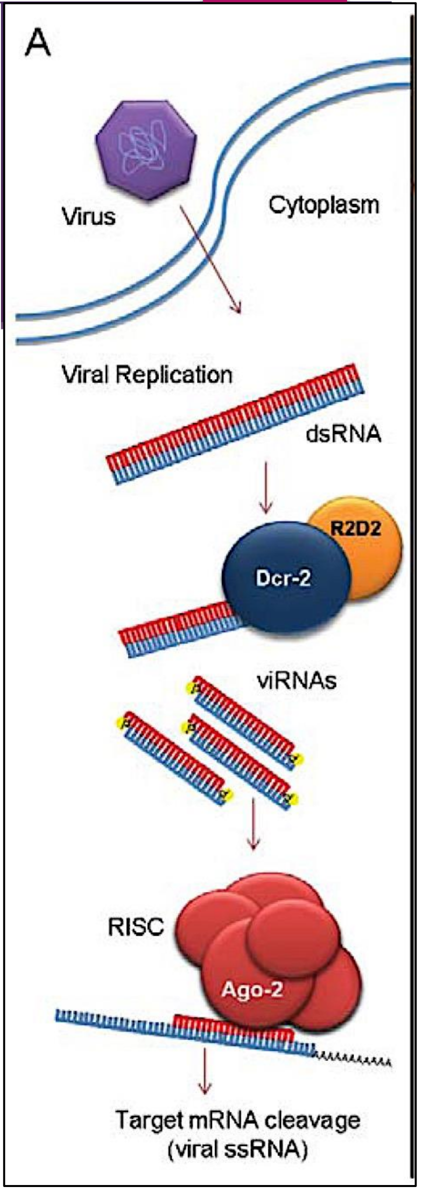
# ➢ Feijões resistentes a vírus transmitido por mosca brancaC

- A vantagem da técnica é que **não há produção de novas proteínas nas plantas**, não havendo a possibilidade de alergenicidade e toxidez. **Os pesquisadores da Embrapa construíram um vetor para a geração de plantas resistentes com o objetivo de bloquear a multiplicação do vírus do mosaico do feijoeiro (Rep protein)**, com base no conceito do RNA de interferência para silenciar o gene viral.

# ➢ Feijões resistentes a vírus transmitido por mosca brancaC


Ivo Viu a Uva – www.ivoviuauva.com.br

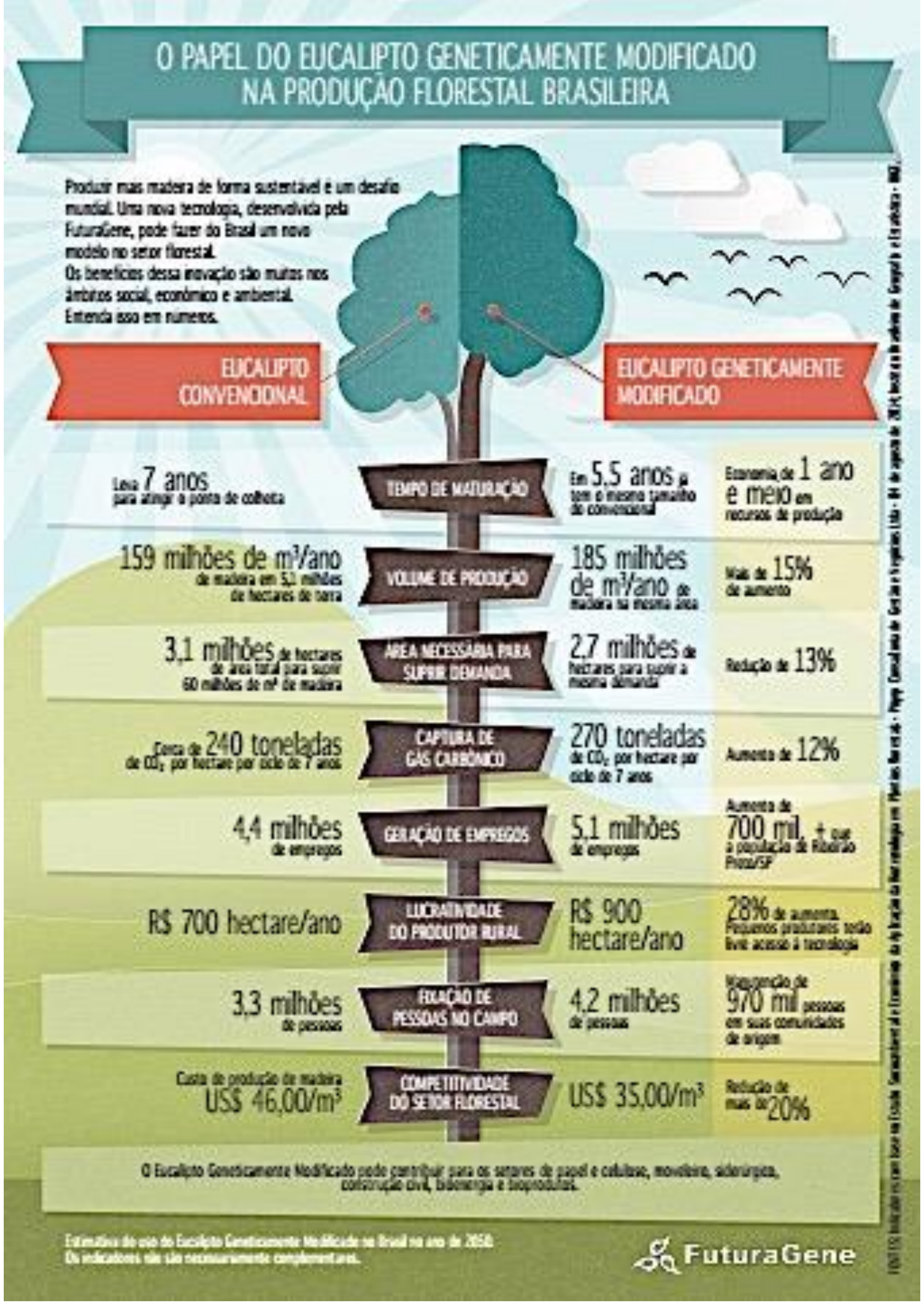
- RNA silencing is a natural defense mechanism against viruses. It is triggered by the processing of virus-derived dsRNA by host Dicer-like (DCL) enzymes into 21- to 24-nucleotide (nt) small interfering RNAs (siRNAs). Viral siRNAs bind argonaute proteins and guide the resulting RNA-induced silencing complexes toward their cognate RNA molecules, leading to posttranscriptional or transcriptional silencing in the case of RNA or DNA viruses, respectively.
    - Mol. Plant-Microbe Int. (2016)



# ➢ Eucaliptos transgênicos

- Modificação do *Eucalyptus* visando ao aumento volumétrico de madeira
- Liberado pela CTNBio em 2015, empresa Futuragene
- **Evento H421 de *Eucalyptus grandis* x *E. urophylla***

- Expressa o produto do gene *cell*, isolado de *Arabidopsis thaliana*, que é a enzima 1,4-B-endoglucanase, a qual se liga à celulose e às xiloglucanas, promovendo o relaxamento da parede durante a fase de alongamento celular. A expressão desta cópia funcional da 1,4-B-endoglucanase acelera o crescimento da árvore, havendo um aumento do volume de biomassa por área.


O PAPEL DO EUCALIPTO GENETICAMENTE MODIFICADO
NA PRODUÇÃO FLORESTAL BRASILEIRA
Produzir mais madeira de forma sustentável é um desafio mundial. Uma nova tecnologia, desenvolvida pela FuturaGene, pode fazer do Brasil um novo modelo no setor florestal.
Os benefícios dessa inovação são muitos nos âmbitos social, econômico e ambiental.
Entenda isso em números.
EUCALIPTO CONVENCIONAL
EUCALIPTO GENETICAMENTE MODIFICADO
Leva 7 anos para atingir o ponto de colheita
TEMPO DE MATURAÇÃO
Em 5,5 anos já tem o mesmo tamanho do convencional
Economia de 1 ano e meio em recursos de produção
159 milhões de m³/ano de madeira em 5,1 milhões de hectares de terra
VOLUME DE PRODUÇÃO
185 milhões de m³/ano de madeira na mesma área
Mais de 15% de aumento
3,1 milhões de hectares de área total para suprir 60 milhões de m³ de madeira
ÁREA NECESSÁRIA PARA SUPRIR DEMANDA
2,7 milhões de hectares para suprir a mesma demanda
Redução de 13%
Cerca de 240 toneladas de CO₂ por hectare por ciclo de 7 anos
CAPTURA DE GÁS CARBÔNICO
270 toneladas de CO₂ por hectare por ciclo de 7 anos
Aumento de 12%
4,4 milhões de empregos
GERAÇÃO DE EMPREGOS
5,1 milhões de empregos
Aumento de 700 mil, + que a população de Ribeirão Preto/SP
R$ 700 hectare/ano
LUCRATIVIDADE DO PRODUTOR RURAL
R$ 900 hectare/ano
28% de aumento. Pequenos produtores terão livre acesso à tecnologia
3,3 milhões de pessoas
FIXAÇÃO DE PESSOAS NO CAMPO
4,2 milhões de pessoas
Manutenção de 970 mil pessoas em suas comunidades de origem
Custo de produção de madeira US$ 46,00/m³
COMPETITIVIDADE DO SETOR FLORESTAL
US$ 35,00/m³
Redução de mais de 20%
O Eucalipto Geneticamente Modificado pode contribuir para os setores de papel e celulose, moveleiro, siderurgico, construção civil, bioenergia e bioprodutos.
Estimativa do uso do Eucalipto Geneticamente Modificado no Brasil no ano de 2050. Os indicadores não são necessariamente complementares.
FuturaGene

## ➢ Milho tolerante à seca:

Gene ***cspB*** de *Bacillus subtilis*, é uma **cold shock protein (induced stress protein)**: mantém as funções celulares sob estresse hídrico por preservar a estabilidade de RNA e permitir assim a sua tradução.

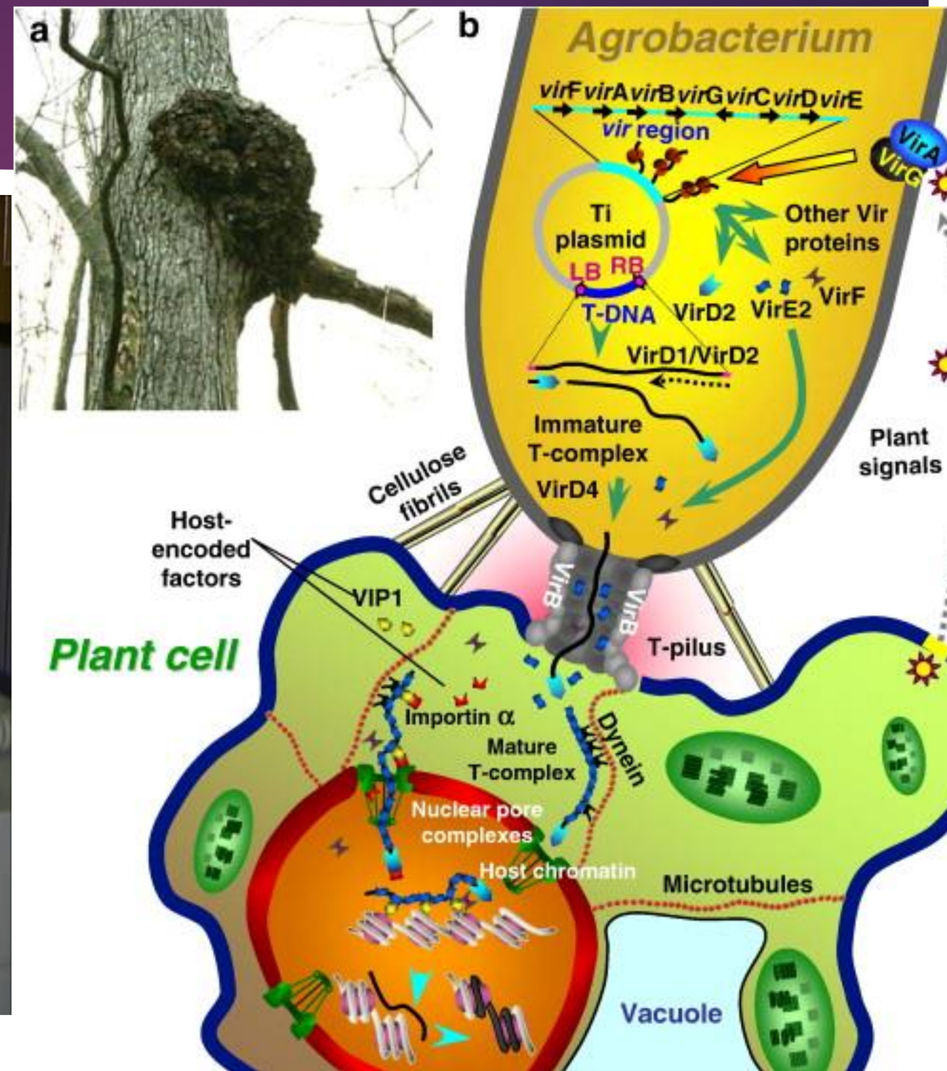
Empresa Monsanto

Willimsky et al. J. Bacteriol. 1992; 174(20): 6326–6335.

- 2016 e 2017: vários eventos combinados
- 2017: cana *Bt*
- 2018: soja

- Cana-de-açúcar geneticamente modificada visando a resistência a insetos: cana Bt, ***gene cry1Ab***
- Centro de Tecnologia Canavieira – CTC

Preference for *Agrobacterium* as a vector ?



# GM Crop report relating to Brazil: 111 events

| Plant | Authorized events | Cultivated area (ha) | Area of GMO cultivation ha | % GM |
|---|---|---|---|---|
| Bean (**BRS FC401 RMD**) | **1** | | Not cultivated | |
| Cotton | **23 (14)** | 927,200 | 726,000 | 78.3 |
| Maize | **64 (35)** | 16,030,000 | 12,200,000 | 82.0 |
| Soybean | **19 (11)** | 32,700,000 | 31,065,000 | 96.5 |
| Eucalyptus (**H421**) | **1** | | Not cultivated | |
| Sugarcane | **3** | | Not cultivated | |
| Total | **111** | | **43,991,000** | |

✔ **Compulsory labeling for food and feed** ✔ **Last update: 2020/Sep**

# Culturas agrícolas GM aprovadas no Brasil: 111 eventos

- http://www.isaaa.org/gmapprovaldatabase/approvedeventsin/default.asp?CountryID=BR&Country=Brazil

- http://ctnbio.mcti.gov.br

# Commercial GM Traits List

- Abiotic Stress Tolerance: **Genuity® DroughtGard™ maize and Verdeca HB4 Soybean**
- **Altered Growth/Yield: GM Eucalyptus**
- **Disease Resistance: *Phaseolus vulgaris (1), Carica papaya (4), Prunus domestica (1), Solanum tuberosum L.. (19), Cucurbita pepo (2), Capsicum annuum (1), Lycopersicon esculentum* (1)**
- **Herbicide Tolerance: *Medicago sativa (4), Argentine Canola - B. napus (34), Cichorium intybus (3), Gossypium hirsutum (45), Creeping Bentgrass - Agrostis stolonifera (1), Linum usitatissimum (1), Zea mays (210), Solanum tuberosum (4), Oryza sativa (3), Glycine max (33), Beta vulgaris* (3), *Nicotiana tabacum* (1) *Triticum aestivum* (1)**
- **Insect tolerance**: ***Gossypium hirsutum (***50) ***Vigna unguiculata*** (1), ***Solanum melongena*** (1), ***Zea mays*** (208) ***Populus sp.*** (2) ***Solanum tuberosum (***30) ***Glycine max (***6), ***Saccharum sp*** (3), ***Lycopersicon esculentum*** (1)
- Pollination control system
- Modified Product Quality

**Event Name: 55-1**
**Trade Name: Rainbow, SunUp**
Crop: *Carica papaya* - Papaya
**Developer:** Cornell University and University of Hawaii
**Method of Trait Introduction:** Microparticle bombardment of plant cells or tissue
**GM Trait s:** Viral disease resistance , Antibiotic resistance , Visual marker
**Commercial Trait:**
(Singular) Disease Resistance

# Summary of Basic Genetic Modification
## * : Selection Marker/Reporter

| Gene Introduced | Gene Source | Product | Function |
|---|---|---|---|
| prsv_cp | Papaya ringspot virus (PRSV) | coat protein (CP) of the papaya ringspot virus (PRSV) | confers resistance to papaya ringspot virus (PRSV) through "pathogen-derived resistance" mechanism |
| nptII * | *Escherichia coli* Tn5 transposon | neomycin phosphotransferase II enzyme | allows transformed plants to metabolize neomycin and kanamycin antibiotics during selection |
| uidA * | *Escherichia coli* | beta-D-glucuronidase (GUS) enzyme | produces blue stain on treated transformed tissue, which allows visual selection |

- CTNBio:
- Como proceder a avaliação de risco de plantas cujo genoma é modificado usando as novas tecnologias de edição?
- Não há risco, pois não há inserção de transgenes (?)


*Vamos caminhando...*